TITLE

ABCB6 Polymorphisms are not Overly Represented in Patients with Porphyria

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Word Count:
Title: 73 with spaces
Short Title: 47 with spaces
Abstract: 197
Text: 3018 (not including the abstract)
References: 27
Figures: 1
Tables: 2

Supplemental Material:
Figures: 2
Tables: 3

Short title: ABCB6 polymorphisms not associated in porphyria

Key points: #1- ABCB6 is located on multiple organelles and the cell surface, the range of substrates transported has not been defined. #2 In all types of porphyria, ABCB6 polymorphisms are not overrepresented when compared to the overall population.

ABSTRACT
The Mendelian inheritance pattern of acute hepatic porphyria, hereditary coproporphyria, and variegate porphyria is autosomal dominant. The penetrance within the general population is low, and the clinical phenotype is heterogeneous, but among first-degree relatives of a proband, penetrance is higher. These observations suggest that genetic factors, in addition to mutation of the specific enzyme of the biosynthetic pathway of heme, contribute to the clinical phenotype. Recent studies by others suggested that the genotype of the transporter protein ABCB6 contribute to the porphyria phenotype. Defining the molecule(s) that are transported by these ABC type transporters has been difficult and has led to some confusion with regard to how variants/mutations in the sequence lead to the varied and diverse phenotypes. Knockout mouse models of *Abcb6* have not provided clear direction since these mice do not express a definitive phenotype associated with homozygous loss. To address the proposed link to porphyria studies of a large cohort of patients with acute hepatic porphyria and erythropoietic protoporphyria were performed. We were unable to demonstrate that *ABCB6* genotype correlates with disease severity. Our findings suggest that genotyping of *ABCB6* in patients with acute hepatic porphyria is not warranted.

INTRODUCTION

The acute hepatic porphyrias (AHPs) is a term used to include; acute intermittent porphyria (AIP); hereditary coproporphyria (HCP); and variegate porphyria (VP) these are rare diseases of heme biosynthesis that are inherited in an autosomal dominant fashion with low penetrance.[1] For example, AIP, the most common of the AHPs, arises as a consequence of mutations affecting the enzyme hydroxymethylbilane synthase (HMBS) that is required to convert porphobilinogen to hydroxymethylbilane (the third of eight steps in the biosynthetic pathway of heme). In a study of genomic/exomic databases, Chen and colleagues[2] identified 12 pathogenic sequence variants among ~92,000 Caucasian alleles, a frequency of 0.00056. The estimated prevalence of symptomatic Europeans with heterozygous mutant HMBS is ~0.000005. Therefore, the estimated penetrance of pathogenic HMBS mutants in Caucasians is ~1%.[3] Among first-degree relatives of affected patients, the penetrance is ~20%.[4] Together, these data suggest that genetic factors, in addition to mutant HMBS, determine the clinical phenotype of AIP.

The basis of the low penetrance observed in AHPs is an area of active investigation, as discovery of genes that modify the disease phenotype would provide both relevant clinical information and a more detailed understanding of heme metabolism.

Steps in the heme biosynthetic pathway take place in both the mitochondria and the cytosol. Therefore, genes that encode proteins that transport heme metabolites are logical targets to investigate for sequence variants that contribute to the disease heterogeneity characteristic of porphyria. Krishnamurthy and colleagues reported that ATP-binding cassette B6 (ABCB6) is a mammalian mitochondrial porphyrin transporter.[5] In unrelated studies, ABCB6 was shown to specify the Langereis (Lan) blood group system.[6] Fukuda et al. hypothesized that sequence variants affecting ABCB6 modulate the clinical phenotype of porphyria by altering the transport of toxic metabolic intermediates.[7] Those investigators reported data suggesting that sequence variants in *ABCB6* were causally related to disease severity in patients of European descent with AHP or erythropoietic protoporphyria (EPP).[7] The cohort of patients examined by Fukuda et al., however, was small (total of 36 patients). To investigate further the relationship between ABCB6 and the severity of the AHPs, we studied the gene sequence in 557 patients (1114 alleles). Our findings demonstrate that with one exception ABCB6 variants are present at the same frequency in patients with AHPs and EPP as in the general populations and when combined there is no correlation between ABCB6 genotype and porphyria phenotype.

Materials

A retrospective study was performed on DNA samples obtained from patients with Acute Intermittent Porphyria (AIP), Hereditary Coproporphyria (HCP), Erythropoietic Protoporphyria (EPP), and X-linked Protoporphyria (XLP). The samples were collected under studies approved by the local Institutional Review Boards of the 6 academic medical centers of the Porphyrias Consortium or the French Porphyria Group. All research was performed in accordance with the World Medical Association Declaration of Helsinki ethical principles for medical research involving human subjects and its subsequent amendments (R162-16-7 and 145-15-4 French ethical agreement). All family members provided written informed consent to participate in the genetics studies. The European Union Institutional Review Board at INSERM approved the study protocols.

Methods

Genomic DNA (gDNA) was isolated from peripheral blood mononuclear cells using an automated magnetic bead-based system (PerkinElmer Chemagic 360). Briefly, whole blood was transferred to 50 mL conical tubes containing 50 uL of protease and 900 uL magnetic beads that bind DNA. Lysis buffer, binding buffer, and several wash buffers were sequentially dispensed by the machine during the extraction run. Samples of DNA were removed from beads with 1 mL of elution buffer (10 mM Tris HCL, pH = 8.0) and the sample was incubated at 65° C for 1 hour. DNA was quantified using a Nanodrop One spectrophotometer (ThermoFisher) and the concentration was adjusted to 200 ng/uL. Samples were run on an agarose gel to ensure that the DNA was high molecular weight.

Sequencing of the French cohort for ABCB6 was performed using primers shown in Supplemental Table 1. All patient sequences were compared to the NCBI Reference

Sequence: NG_032110.1, this sequence was initially described.[6] Allele typing of the American cohort was performed using an ABCB6 SNP detection protocol at the Molecular Diagnostic Laboratory at the Icahn School of Medicine at Mount Sinai; ABCB6 exons were amplified in 3 groups: Ex1-4 (3445 bp); Ex 5-13 (3426 bp); and Ex13-19 (3435 bp). All PCR primers and sequencing primers (Supplemental Table 2) were designed in-house using the MacVector software and checked for common SNPs by the SNP-check program (http://www/ngrl.org.uk/Manchester/projects/snpcheck). PCR amplification was performed with the Takara PrimeStar GXL DNA Polymerase kit from Clontech Laboratories using the Bio-Rad C1000 Touch Thermal Cycler. ABCB6 exons were sequenced bi-directionally. Sequencher software (Gene Codes Corporation) was used for alignment and analysis of the traces and SNPs identification.

All allele frequencies used are taken from the ExAC database as of January 3, 2019. Comparison of allele frequencies between groups was performed using Fisher's Exact Test.[8] Differences were considered as significant if the p-value was equal to or lower than 0.0045. In cases where all 11 polymorphisms were examined across the genome then the significance of the result is compounded by the number of tests performed and the method of Bonferroni correction is used and p-value of 0.05/11=0.0045 and a p-value less than or equal to this threshold was considered significant.

Results

Sequencing of ABCB6 Allele Variants

We sequenced the gene encoding ABCB6 in 557 subjects with various forms of porphyria, for a total of 1114 alleles. Subjects with a confirmed molecular diagnosis were divided into those with severe disease (216 patients) or as mild/asymptomatic (341 patients), (Table 1). AIP subjects (allele variant in *HMBS*) were divided into those with severe disease (67 patients), experiencing more than 4 acute attacks per year or as mild/asymptomatic (155), defined as <2 acute attacks per year. In the mild/asymptomatic cohort there were an additional 105 individuals identified through family studies that had minimal or no symptoms of AIP.

The cohort contained a total of 49 subjects with HCP, 15 presenting with a much more severe clinical phenotype and 34 with less severe attacks. The combined cohort also included 125 subjects with VP, 46 with severe VP and 79 with a mild clinical presentation.

There were 30 subjects with EPP, of those, half were classified with a mild disease based on the frequency and severity of symptoms. Five subjects were included that had the X-linked form of EPP, XLP. In XLP, gain of function mutations in the ALAS2 gene that increase activity 2-3 fold[9], lead to increased levels of PPIX and Zn-PPIX in the erythroid system. Five subjects were also included in the analysis that had biochemically confirmed increases of PPIX and Zn-

PPIX consistent with EPP where no genetic alteration could be found in any of the genes known to cause EPP.

Eleven sequence variants were identified in *ABCB6* in the 1114 alleles examined, 10 are classified as missense mutations and one is a newly identified synonymous variant. This newly identified single nucleotide polymorphism leads to a synonymous change in codons used for Arginine and was identified on a single allele from a patient with AIP, (R589R, c.1767 T>C). Of the 1114 alleles sequenced, 86 missense alterations were identified in the ten different polymorphic variants (Table 2).

ABCB6 Variants in Acute Intermittent Porphyria

There were 222 subjects with a diagnosis of AIP, 67 were classified as severe and 155 were classified as mild (Table 1). In the severe AIP group, there were 134 alleles sequenced, 11 ABCB6 variants were identified in six of the 10 missense alleles (Table 2). In the mild AIP group, the 310 alleles sequenced had 29 ABCB6 variants in nine of the missense alleles. None, of the identified ABCB6 variants were associated with disease severity(p=0.21-1.00).

Two of the missense alleles identified, one at the codon for arginine at position 192, (R192W), and the second at glycine 588 (G588S) were significantly overrepresented in all AIP subjects when compared to the allele frequencies with those reported in the ExAC database (p=0.0038, p=0.0042 respectively) (Table 2). The presence of a missense allele did not correlate with the levels of ALA and/or PBG, and the frequency of the missense alleles was not significantly different between those patients with mild vs severe disease. Missense alleles were identified in nine other positions in ABCB6; however, none were overrepresented when compared to the ~120,000 alleles sequenced in the ExAC database.

Allele frequency in EPP

There were 166 subjects with an EPP phenotype, 85 with a severe EPP phenotype, 3 with XLP presenting with high PPIX and Zn-PPIX. There were 78 subjects with biochemically confirmed EPP that presented with more mild symptoms (less severe sun sensitivity) all but 5 had confirmed genetic validation of EPP (mutations in *FECH*, *ALAS*2, *CLPX*). Missense alleles were identified in 23 of the 332 alleles sequenced. None of the *ABCB6* variants identified were overrepresented in this subset of patients when compared to the allele frequencies in the ExAC database.

RBCs from EPP and XLP patients have a marked increase in fluoresce due to the increased PPIX content (Figure 1). These cells are referred to as fluorocytes and have PPIX levels are in the ~450 μM range, when analyzed by HPLC. In comparison, the level of PPIX in RBCs from normal individuals is in the low nM range. The concentration of PPIX in the plasma of patients with EPP is approximately 0.350 nM (+/- 73) and 0.423 nM in patients with XLP (+/- 149).[10]

The vast majority of these patients are wild type at both *ABCB6* loci and none are homozygous null or compound heterozygotes for any of the variants in *ABCB6*. In the EPP/XLP cohort the cellular concentration of PPIX is approximately 0.5 mM and the plasma PPIX is 0.5 nM. This suggests that if PPIX transport by ABCB6, it does so inefficiently.

Discussion

The "porphyrias" each have their unique pathology due in large part to the intermediate that accumulates when one of the enzymatic steps in the pathway go awry. In AIP, an autosomal dominant disease, acute attacks are characterized by increased the levels of ALA and PBG in the urine. ALA is produced in the mitochondria and then transported into the cytosol for formation of PBG. During an acute attack, levels of PBG are generally in the range of 20-300 mg/g creatinine (N= 0-2 mg/g creatinine) with ALA approximately half the value. Values during an attack are 10-150 times above normal.[11] The values decrease between attacks, however, values often remain elevated above normal for several months to years.[12] During an acute attack, there can also be a modest increase in urinary uroporphyrin in the range of 20-200 ug/g creatinine (< 10X normal) excreted in urine. On successful treatment of the acute attack uroporphyrin values typically returns to normal, 0-30 ug/g creatinine and the levels in the asymptomatic carrier are generally in the normal range.[13]

HCP is an characterized by significant increases in fecal coproporphyrin III. Urinary increases in coproporphyin III are also present. Clinically patients present with acute attacks nearly indistinguishable from AIP, however, an associated photosensitivity is also observed with HCP due to the circulating plasma porphyrins.[1] In VP PPIX accumulates in the plasma, and acute attacks are similar to AIP. The accumulation of PPIX leads to the observed photosensitivity that is similar to EPP.[1]

Phenotypically, erythropoietic protoporphyria is characterized by an accumulation of PPIX in RBCs that is released into the plasma. On exposure of skin to sunlight, the increased plasma PPIX produces a painful photosensitivity.[14,15] Inflammation and the severe pain gradually decrease in 48-72 hours. [11] Multiple genetic causes have been linked to PPIX accumulation including mutations in the gene encoding FECH, the erythroid form of ALA-synthase (ALAS2), the mitochondrial CLPX, an AAA-ATPase family member that is needed to insert the pyridoxal phosphate cofactor (PLP) into ALAS and then ultimately degrade the ALAS protein.[16] Mutations in FECH account for 85-90% of all cases of EPP. The X-linked form of EPP, XLP, accounts for approximately 10% of cases and in the remaining 1-2% of cases, the genetic basis is not known.[17]

When FECH is rate-limiting there is an accumulation of PPIX due to the enzymatic limitation of inserting ferrous iron into the PPIX substrate, to form heme. In XLP, nonsense mutations in *ALAS2* truncate the protein in the last 18-25 residues of the ALAS2 protein.[9] These gain of function mutations that lead to an approximate 2-3 fold increase in enzymatic activity.[18] [19] The RBCs of these subjects accumulate PPIX because FECH now become rate-limiting with the added flux through the pathway. In XLP patients there is accumulation of PPIX but also significant increases in Zn-PPIX when compared to EPP patients.

The compound(s) that is transported by ABCB6 has not been identified. The location of the protein varies in different cell types and loss of ABCB6 in mouse and man have minimal phenotypic consequences. [6] Specific identification of a defect in cells or animals lacking ABCB6 suggests that the compound(s) transported are either essential and as such other transporters accommodate the transport need in situations of loss; or that the transporter is needed for a very specific metabolite that is non-essential for viability in cells or organisms.[20,21] Once a true biologic substrate has been identified it may be possible to define a stress situation that uncovers the essential nature of ABCB6. There are several studies that link ABCB6 to heme biosynthesis, however, it has not been consistently localized to the mitochondria.[5,20-24] This is important in that the initial step and the final 3 steps in heme biosynthesis take place in the mitochondria with enzymatic steps 2-5 being catalyzed in the cytosol.[25] Many of the enzymes in heme biosynthesis are reported to be expressed with the same temporal pattern as *ABCB6.*[7] Recent studies show that heme biosynthesis requires a large shift in metabolic patterns within the developing erythron. Feeding appropriate abundant intermediates, such as α-ketoglutarate into the tri-carboxylic acid (TCA) cycle for the formation of succinyl-CoA, a required metabolic building block of δ-aminolevulinic acid, has been shown.[26] This requires that a different set of host proteins be produced, it is very possible that ABCB6 is required for amino acid balancing rather than transporting direct intermediates of heme biosynthesis.[26] More recent studies have shown that ABCB6 is necessary for proper melanin production.[24] These studies once again lack identification of the substrate, but it is clear that loss of ABCB6 contributed to decreased pigment production in cells. Localization of ABCB6 to the endosomal compartment in this system suggests that the transport role is not for an intermediate of heme biosynthesis since none of the 8 reactions to form heme occur in the endosomal compartment.

One of the key experiments that suggests that ABCB6 is not transporting tetrapyrrole intermediates in heme biosynthesis comes from patients with EPP/XLP. As was pointed out by Helias et al. in erythrocytes of subjects that have the Langereis (Lan) blood type, there is loss of ABCB6 on the plasma membrane with no observable phenotype other than complications with transfusions due to the production of antibodies to ABCB6 by Lan-/- recipients.[6] Any excess PPIX produced in the process of hemoglobinization, would be expected to be present in the RBCs of patients with the Lan blood type, this was not observed.

The observation that polymorphisms exist in *ABCB6* in patients with different types of porphyria was not supported by the 1114 alleles we examined. What accumulates in each of these different porphyrias is not just the substrate of the defective enzyme. In cases of the acute hepatic porphyrias, due to enzyme defects in steps 2, 3, 6 or 7 (ALA-dehydratase, hydroxymethylbilane synthase, coproporphyrinogen oxidase and protoporphyrinogen oxidase respectively), multiple substrates accumulate. There were no significant differences in the frequency of mutations in the severe vs mild forms of porphyria, providing evidence that ABCB6 function does not modify severity of disease or frequency of porphyric attacks. It is not clear why enzymatic defects in downstream steps lead to accumulation of ALA and PBG, however, the frequency of *ABCB6* polymorphisms does not correlate with severity.

The PPIX accumulation phenotype and liver architecture was examined in multiple mouse strains [27] and significant differences were identified that suggested additional genetic modifiers were involved in where PPIX accumulated and to what level the PPIX did accumulate. Intercrossing mice with different PPIX phenotypes, each with unique factors to score, did not identify allelic segregation patterns in the F1 or F2 hybrid generations that mapped to the *Abcb6* locus in these progeny (data not shown). Several genetic loci were highly ranked, which suggests that hepatic uptake and processing of PPIX from the plasma to the bile is modulated by other genetic factors. However, *Abcb6* was not a candidate identified by these methods. This conclusion is also supported by the phenotypic characterization of the *Abcb6*-/- mouse that has no apparent defect in tetrapyrrole homeostasis or red blood cell development under normal conditions.[28]

Looking at all of the variants in the ABCB6 gene, and particularly the 11 alleles identified in this combined patient cohort and applying the Bonferroni correction there is no significant difference in the frequency of polymorphisms within the gene. The combination of two cohorts from Europe and the United States, each supporting the conclusion that there is no over-representation of variants in ABCB6 provides additional power over what was seen in a smaller independent population. It is clear from the studies of Fukuda et al., that some of the polymorphisms affect the stability of the protein, however, in the absence of a known transport molecule(s) it is difficult to complete the picture of how this will affect the overall metabolic processes within the cell.[7] There were only two residues that when reviewed individually suggested that the alleles were over represented in the various porphyrias, however, when all alleles were combined there was no over-representation of mutations in *ABCB6* when compared to the general population.[7] In this and the study by Fukuda et.al. the contribution of familial patterns to overrepresentation of any single polymorphism is not accurately accounted for (Supplemental Table 3).[7] It is not clear at this time what the role of ABCB6 is in erythrocyte development, however, it does not appear to directly transport intermediates in the heme biosynthetic pathway.

Acknowledgment:
Research work in the laboratory of H.P., L.G., G.N., and ZK. is supported by the Laboratory of Excellence Gr-Ex (Labex Gr-Ex) and the Institut National de la Santé et de la Recherche

Medicale (INSERM). The Labex GR-Ex is funded by the program “Investissements d’avenir” of the French National Research Agency; reference ANR-11-IDEX-0005-02. Research studies in the laboratory of RJD and BC were supported in part by the Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai and NIH grant U54 DK083909 and the American Porphyria Foundation (APF). Support of CPF, CJP, JDP was from NIH U54 DK118058, U54 DK083909 and the APF.

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Table 1. Cohort of 562 Porphyric patients from the USA (American Porphyria Consortium) and Europe (French Porphyria Center; FCP)

	All Porphyric cohort	AIP		VP		HC		EPP		XLP	
	Total (USA / France)	USA	FPC	USA	FPC	USA	FPC	USA	FPC	USA	FPC
All (n alleles)	1124 (390/ 734)	250	194	4	246	0	98	120	196	6	0
Severe (n alleles)	330 (100/ 230)	22	112	2	90	0	30	60	110	6	0
Mild – Asymptom. (n alleles)	482 (80 / 392)	18	82	2	156	0	68	60	86	0	0
Unknown severity* (n alleles)	210 (210/ 0)	210	0	0	0	0	0	0	0	0	0

AIP: acute intermittent porphyria; VP: variegate porphyria; HC: hereditary coproporphyria; EPP: classical erythropoietic protoporphyria; XLP: X-linked protoporphyria + EPP variant form (CLPX, others).

* known allele carrier from family study

Table 2. Allele frequency of ABCB6 by porphyria subtype comparing severity.

	AIP		HCP		VP		EPP		EPP*	XLP		
	S	M	S	M	S	M	S	M			Total	MAF
Patients	67	155	15	34	46	79	80	73	5	3	557	
Alleles	134	310	30	68	92	158	160	146	10	6	1114	
R192Q	2	5				1	3			1	12	0.00342
R192W	2	1	1	2	1	2	3	2			14	0.00183
R247C		1					1				2	0.00646
R276W	1	9	1		1	1	2	1			16	0.01895
R343Q		1							1		2	0.01911
A492T	2	2		1	1	1	1	2			10	0.00716
T521S	2	2	1	1	1	1	2	1			11	0.00385
G588S	2	7		2	1	3	2	1			18	0.00467
A681T											0	0.00027
G729S		1									1	0.00005
R589R	1										1	0
total VUS	12	29	3	6	5	9	14	7	1	1	87	

* Protoporphyrin accumulation without mutation in FECH, ALAS2, CLPX. The A681T was not identified in this population.

Figure Legend

Figure 1. Peripheral blood smear of control subject (A, B); and patient with erythropoietic protoporphyria (EPP) (C, D). In panels A and C DIC and fluorescence is shown as an overlay; in panels B and D only, fluorescence is shown. The fluorescent protoporphyrin is visualized by excitation at 402 nm reading the emission at 600-650 nm. Note, protoporphyrin is present only in RBCs, the lymphocytes present have no accumulated protoporphyrin.